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Rapid report

Non-conventional modification of low density lipoproteins: chemical models for macrophage recognition of oxidized LDL

George Hoppe [a], June O'Neil [a], Lawrence M. Sayre [b,1], Henry F. Hoff [a,*]

[a] *Department of Cell Biology, NC-10, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, USA*
[b] *Department of Chemistry, Case Western Reserve University, Cleveland, OH 44106, USA*

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Abstract

To define the structural and chemical criteria governing recognition of oxidized LDL (oxLDL) by mouse peritoneal macrophages (MPM), we exposed LDL to novel chemical modification agents that induce defined neutralizing and non-neutralizing alterations of lysine as models for distinct apoB adducts present in oxLDL. We found some exceptions to the usual notion that neutralization of lysine positive charges is the principal determinant governing MPM recognition. In addition, competitive binding experiments using chemically modified ^{125}I-LDL preparations revealed that, whereas some modifications engendered recognition principally by the classical scavenger receptor class A (SRA), as seen for acetylated LDL (acLDL), chemical models of advanced aldehydic modifications of LDL led instead to MPM uptake mainly by oxLDL receptors distinct from SRA. © 1997 Elsevier Science B.V.

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Oxidation of low density lipoprotein (LDL) has been implicated in the pathogenesis of atherosclerosis [1,2]. The link is based on the observation that oxidized LDL (oxLDL) is internalized by a class of high affinity receptors on macrophages that include the scavenger receptor class A type I/type II (SRA) [3–6] which is not downregulated by the intracellular levels of cholesterol, and therefore leads to lipid loading of such macrophages called foam cells [1–3,6]. Such cells are present in the intima of large arteries and constitute the characteristic of the fatty streak lesion [7]. The prototypic modification leading to SRA recognition is acylation (e.g., acetylation, maleylation, succinylation) [3,5,8] or carbamylation [9], which induce large increases in the relative electrophoretic mobility (REM) of the LDL particles on account of neutralization of lysines [5,10]. By contrast, reductive methylation, which leaves the LDL lysine charge unaffected [11], results in little change in REM and no significant SRA recognition [3]. It

Abbreviations: acLDL: acetylated LDL; anLDL: acrylonitrile-treated LDL; cmLDL: carbamylated LDL; hdLDL: 2,5-hexanedione-treated LDL; HNE: 4-hydroxy-2-nonenal; LPDS: lipoprotein-deficient serum; LDL: low density lipoprotein; MDA: malondialdehyde; mdaLDL: malondialdehyde-treated LDL; MPM: mouse peritoneal macrophages; onaLDL: 4-oxononanal-treated LDL; oxLDL: oxidized LDL; pdLDL: 2,4-pentanedione-treated LDL; SRA: scavenger receptor type A; TCA: trichloroacetic acid; tmpLDL: 2,4,6-trimethylpyrylium-treated LDL; TNBS: 2,4,6-trinitrobenzene-1-sulfonic acid

* Corresponding author. Tel.: +1-216-444-2248; fax: +1-216-444-9404; e-mail: hoffh@cesmtp.ccf.org.

[1] For correspondence on chemistry: Tel.: +1-216-368-3704; fax: +1-216-368-3006; e-mail: lms3@po.cwru.edu.

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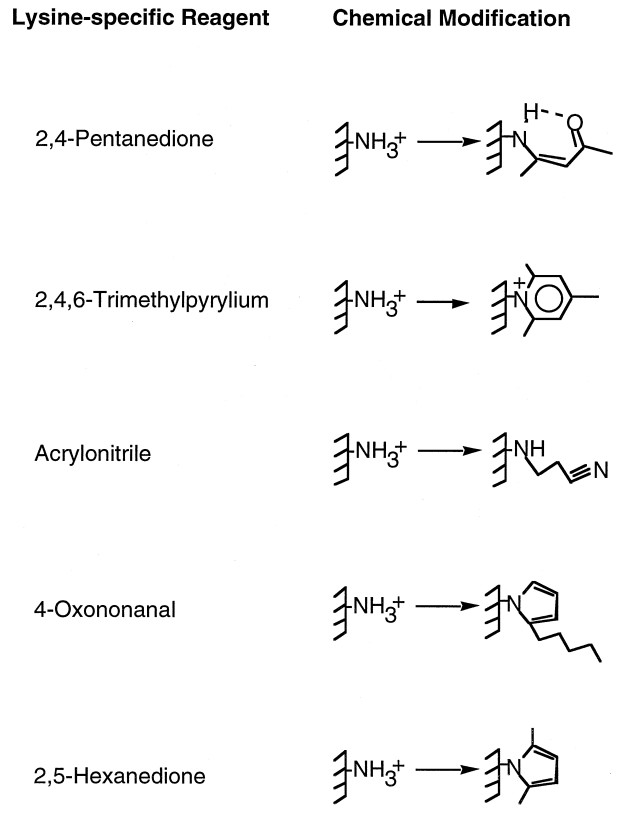
was concluded from these earlier studies that receptor recognition reflects mainly the increase in surface negative charge induced by neutralization of positively charged lysine ε-amino groups. However, these prototypic modifications may not be particularly relevant to chemical changes that occur physiologically in oxLDL [5], and, especially for acylation, may reflect modification of lipoprotein nucleophiles distinct from apoB lysines.

The key modifications present in oxLDL responsible for macrophage receptor recognition are those induced on apoB lysines by the variety of reactive aldehydes generated during peroxidation of LDL lipids [1,2,5,10]. 4-Hydroxy-2-nonenal (HNE) adducts present in oxLDL [12] are thought to be a major actor in cytotoxicity [10,13] and lipid loading [14], though modifications by malondialdehyde (MDA) as well as by α,β-unsaturated aldehydes [15,16] and 3,4-epoxy-2-enals [17] are likely to contribute to the functional properties of oxLDL. Definition of the chemical type(s) of modification most responsible for macrophage uptake of LDL treated with HNE or MDA has not been possible, however, because these aldehydes induce a heterogeneous array of lysine modifications [5,10,18]: MDA can form neutral dihydropyridine adducts, simple lysine enaminones, and enaminoimine lysine–lysine crosslinks [8,10,19], whereas HNE can form both Schiff base and Michael adducts initially, followed by several possible secondary reactions reflecting condensative, dehydrative, and oxidative maturing of the initial adducts [10,20–23]. Since all these adducts are physicochemically quite distinct from acylation and carbamylation, they might thus lead to receptor recognition in ways not seen in previous studies [4,5,8]. We have therefore investigated LDL derivatization by selected lysine group-specific reagents (see Scheme 1) as models for several different *general* types of apoB modification suspected in physiological oxLDL that cannot themselves be generated homogeneously by direct treatment with any of the lipid-derived aldehydes so far identified.

With the exception of 4-oxononanal, synthesized as described elsewhere [22], chemical reagents were used as obtained commercially. Lipoprotein-deficient serum (LPDS) and LDL were isolated, LDL was labelled (with $Na^{125}I$) and oxidized, and protein was determined as described elsewhere [24]. Acetylation of LDL was performed by repeated additions of acetic anhydride [3]. Carbamylation of LDL was achieved by incubation with potassium cyanate [11]. Malondialdehyde modification of LDL was performed as described by Haberland et al. [8]. Novel modifications of ^{125}I-LDL were conducted at 37°C in 0.1 M borate buffer with the chemical reagents selected on the basis of their ability to induce the lysine modifications shown in Scheme 1. Since the different agents possessed different reactivities, we initially carried out modifications at several concentrations, pH values, and time intervals. For comparison purposes, we selected representative treatments required to induce moderate (31–54%) modification of lysyl residues (see Table 1) as determined using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) colorimetric assay [25]. These conditions were: 100 mM 2,4-pentanedione for 21 h (pdLDL), 100 mM acrylonitrile for 21 h (anLDL), 200 mM 2,5-hexanedione for 48 h (hdLDL), and 0.5 mM 4-oxononanal for 4 h (onaLDL)



Scheme 1. Chemical modifications that model types of adduction chemistry likely to occur as a result of oxidative modification of LDL (see text for details).

Table 1
Extent of modification of LDL and recognition by MPM

Modified LDL	Lysine blocked (%)	REM (relative to LDL)	Degradation (μg/mg cell protein)	Association (μg/mg cell protein)
^{125}I-LDL	0	1.0	0.49 ± 0.05	0.17 ± 0.07
^{125}I-acLDL	38	4.0	8.77 ± 0.73	1.23 ± 0.10
^{125}I-cmLDL	34	4.0	11.05 ± 1.80	0.95 ± 0.26
^{125}I-mdaLDL	50	4.0	6.68 ± 0.43	0.86 ± 0.02
^{125}I-pdLDL	51	2.7	3.46 ± 0.08	0.34 ± 0.02
^{125}I-tmpLDL	48	1.2	7.48 ± 0.52	1.26 ± 0.17
^{125}I-anLDL	47	2.6	1.02 ± 0.02	0.36 ± 0.02
^{125}I-hdLDL	31	1.9	10.13 ± 0.34	1.23 ± 0.09
^{125}I-onaLDL	54	2.2	10.73 ± 0.16	1.83 ± 0.02

Aliquots of ^{125}I-LDL (500 μg/ml) were subjected to acetylation (acLDL), carbamylation (cmLDL), MDA treatment (mdaLDL), as described elsewhere [3,8,11], or treated with 2,4-pentanedione (pdLDL), 2,4,6-trimethylpyrylium (tmpLDL), acrylonitrile (anLDL), 2,5-hexanedione (hdLDL), or 4-oxononanal (onaLDL) as described in the text. Lysine blockage [25] and REM [18] were measured as described previously. Macrophage recognition was assessed by incubating cultures of MPM for 5 h at 37°C in RPMI-1640 medium containing 10% LPDS, and one of the modified forms of ^{125}I-LDL (10 μg/ml), followed by measuring cell-degraded and cell-associated ^{125}I-lipoprotein as described in the text.

at pH 8.0, and 20 mM 2,4,6-trimethylpyrylium for 4 h at pH 8.5 (tmpLDL). At the end of each modification procedure, modified forms of LDL were extensively dialyzed against PBS containing 0.3 mM EDTA, centrifuged for 30 min at $10{,}000 \times g$, and passed through a 0.22 μm filter. The electrophoretic mobility relative to LDL (REM) was determined [18] as an indirect measure of the change in charge on the LDL particle as a consequence of lysine modification.

The modified LDL preparations were then incubated with thioglycollate-elicited mouse peritoneal macrophages to determine cell association and degradation values by the procedures described elsewhere [26,27]. In brief, cells were washed with PBS and the indicated concentrations of ^{125}I-ligands were added for 5 h in media containing 10% LPDS. The media were then removed and assayed for trichloroacetic acid (TCA)-soluble, non-iodine degradation products as described previously [26]. Cells were washed three times with PBS, dissolved in 0.1 N NaOH, and assayed for cell-associated label and protein content. Total uptake was calculated from the sum of degradation and cell-associated label. All determinations are reported as mean $\pm$ S.D. of triplicate determinations.

Three modifications of LDL which neutralize lysine charge, acetylation (acLDL), carbamylation (cmLDL), and MDA treatment (mdaLDL), resulted in large increases in REM and substantial uptake/degradation (Table 1), consistent with earlier reports [3,8,9,18]. Since MDA modification includes dihydropyridines and enaminoimine crosslinks as well as Schiff base-derived enaminones, we used 2,4-pentanedione (pdLDL), the diketone analog of MDA known to produce only the latter (Scheme 1, Ref. [28]), as our model of simple Schiff base adduction. Interestingly, this neutralizing modification was associated with only modest increases in REM and levels of uptake/degradation by MPM (Table 1).

Acrylonitrile modification of LDL (anLDL) was used as a model for the Michael type adducts [29] formed between lysine and lipid derived 2-enals [30] and 4-hydroxy-2-enals which decrease the nitrogen pK_a, thereby increasing the degree of neutralization at physiological pH (Scheme 1). Modification by acrylonitrile to an extent of 47% blockage of apoB lysines, resulted in an increased REM, consistent with at least partial lysine neutralization, but only a marginal level of uptake and degradation by MPM (Table 1). These findings suggest that Michael adducts do not contribute significantly to uptake of oxLDL by macrophages. This is one example in which charge neutralization does not guarantee significant binding and uptake by macrophages.

Reductive methylation of LDL lysines is a non-neutralizing modification that fails to induce recognition by scavenger receptors [3]. To ascertain the generality of this finding, we modified LDL with

2,4,6-trimethylpyrylium (tmpLDL), which converts the lysine ε-amino group to a pyridinium group with retention of the positive charge [31] (Scheme 1). The pyridinium adduct served as a model for the nucleus of crosslinks possibly resulting from multistep condensation/dehydration chemistries following initial Schiff base modification by bifunctional aldehydes such as HNE and MDA [10,32,33], similar to what occurs following lysyl oxidase treatment of lysine-rich proteins such as collagen [34]. Evidence that such adducts may be present in oxLDL is the characteristic fluorescence exhibited by atherosclerotic plaques [35] that is also seen in oxLDL at higher oxidation levels [10,13] and upon extended treatment of LDL with HNE [13,14]. Consistent with the expected chemical change [31], 2,4,6-trimethylpyrylium modification led to significant blockage of lysyl residues on apoB without significant changes in REM. Unlike reductive methylation, however, tmpLDL induced significant levels of uptake and degradation by MPM (Table 1). Thus, it does not appear that the lack of receptor recognition accompanying reductive methylation is paralleled by all lysine modifications which retain the charge.

A further novel modification of LDL reported in this study involves γ-dicarbonyl reagents which convert the lysine amino groups to neutral pyrroles [21,36]. 2,5-Hexanedione (hdLDL) forms a 2,5-dimethylpyrrole, while 4-oxononanal (onLDL) forms the same 2-pentylpyrrole (Scheme 1) that we showed previously to represent a minor, yet important late-stage stable adduct that arises when proteins are treated with HNE [37]. We refer to the 2-pentylpyrrole as an 'advanced lipid peroxidation end product' (ALE), analogous to advanced glycation end product (AGE) modifications. Achieving similar levels of lysine modification required higher concentrations of the diketone 2,5-hexanedione than the ketoaldehyde 4-oxononanal (see Section 2), which was anticipated on the basis of their relative chemical reactivities [38]. Nonetheless, at roughly equivalent levels of modification, practically identical levels of uptake and degradation were induced by either reagent (Table 1). This result is consistent with the hypothesis that 2-pentylpyrrole formation in HNE-modified LDL may be a key contributor to macrophage uptake and degradation of oxLDL. Based on the modest amounts needed to induce high values of degradation, 4-oxononanal appears to be the most potent reagent inducing macrophage recognition of LDL so far reported, with perhaps the exception of a structurally related modification induced by the oxidative product of arachidonic acid, levuglandin [39].

Based on the data presented in Table 1, those modifications that demonstrated enhanced internalization and processing by MPM were subsequently subjected to competition studies to ascertain whether they were recognized by the SRA [6] and/or via some other receptor(s), previously documented to recognize oxLDL [4,40]. As seen in Fig. 1, degrada-

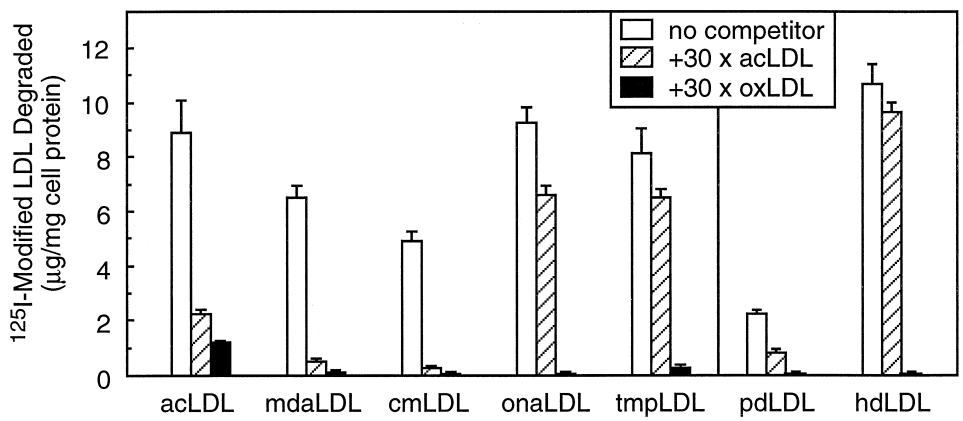


Fig. 1. Competition by excess amounts of unlabeled acLDL or oxLDL for the degradation of modified ^{125}I-LDL by MPM. Each well of a 24-well plate received 250 μl of RPMI-1640 medium containing 10% LPDS, and one of the modified forms of ^{125}I-LDL (10 μg/ml) in the presence or absence of a 30-fold excess of unlabeled acLDL or oxLDL (300 μg/ml). After incubation for 5 h at 37°C, the amounts of degradation were determined as described in the text.

tion of ^{125}I-labelled acLDL, mdaLDL, or cmLDL was essentially completely inhibited by a 30-fold excess of both unlabelled acLDL and oxLDL, consistent with published results [3,8,9]. Excess amounts of unlabelled oxLDL and, for the most part, unlabelled acLDL also competed for the degradation of ^{125}I-pdLDL. The results for cell association of these modified forms of LDL at 37°C mirrored the results on degradation but were about one order of magnitude lower (not shown). That both excess acLDL and oxLDL effectively competed for degradation of ^{125}I-labelled pdLDL, acLDL, mdaLDL, or cmLDL, is consistent with the recognition of all these neutralizing modifications by the SRA [3,8]. By contrast, degradation and cell association of ^{125}I-LDL modified by the two pyrrolylating agents, 2,5-hexanedione and 4-oxononanal, and of the charge-retaining pyridinium modifier, 2,4,6-trimethylpyrylium, was blocked by excess oxLDL, but reduced only slightly (20%) by excess acLDL (Fig. 1). These particular modified LDLs thus appear to share binding site(s) on MPM predominantly with oxLDL, but only slightly with acLDL.

Since we previously showed that oxLDL can significantly inhibit lysosomal proteolysis [24,27], we felt it was necessary to assess whether enzyme inactivation was a potential cause of the uniform inhibition of degradation of the various modified ^{125}I-LDL preparations by oxLDL. We therefore determined the cell association of representative modified LDL preparations at 4°C, which reflects macrophage receptor binding in the absence of any uptake processes. We found that binding at 4°C of hdLDL and tmpLDL was similarly blocked by oxLDL, but only slightly by acLDL (Fig. 2). As this is consistent with results on degradation at 37°C, it is unlikely that the competition seen for degradation of modified LDL by oxLDL was due to lysosomal enzyme inactivation. In further control studies performed using non-lipoprotein ^{125}I-ligands, we verified that oxLDL was not exerting a non-specific effect on all cell receptors (Hoppe et al., unpublished studies). Collectively, these results support our conclusion that uptake/processing of the pyrrole- and pyridinium-bearing-LDLs is mediated largely by an oxLDL-receptor [4] distinct from SRA [6].

In conclusion, our goal in this study was to evaluate uptake of oxLDL by macrophages using chemical

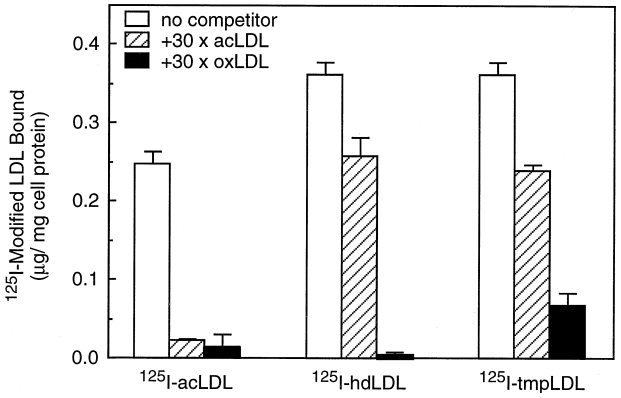


Fig. 2. Competition of excess amounts of unlabeled acLDL and oxLDL for the binding at 4°C of modified ^{125}I-LDL to MPM. Each well of a 24-well plate received 250 μl of RPMI-1640 medium containing 10% LPDS, 20 mM HEPES, and ^{125}I-acLDL, ^{125}I-hdLDL, or ^{125}I-tmpLDL at 10 μg/ml, in the presence or absence of a 30-fold excess of unlabeled acLDL or oxLDL (300 μg/ml). After incubation for 2 h at 4°C, the amount of cell-associated ^{125}I-lipoprotein was determined as described in the text.

modifiers which might be more relevant than acylation to chemical changes expected on oxLDL as a result of lysine derivatization by lipid peroxidation products. The ability of excess amounts of oxLDL but not of acLDL to compete maximally for binding, internalization, and degradation of onaLDL, hdLDL, or tmpLDL in MPM, suggests that the major contribution of recognition of these modified forms of LDL is by a receptor different from the SRA, but one recognizing oxLDL. In the case of onaLDL and hdLDL, the conversion of LDL lysines to pyrroles must thus alter the physicochemical behavior of the LDL particle (presumably its surface) in a manner distinct from that induced by classical neutralizing modifications which require only recognition by the SRA. In the case of tmpLDL, the pyridinium adduct must alter some feature (perhaps conformational and/or local dielectric changes) on the lipoprotein, despite the fact that overall charge is retained in the same manner as for lysine methylation. Substantial current evidence now exists for classes of oxLDL receptors such as CD36 [41] and CD68 [4] that are distinct from the SRA. Our results represent the first clues to the structural nature of the types of lipid-derived modifications present on apoB lysines that engender recognition by non-SRA receptors. Although the charge neutralization hypothesis may define re-

quirements for ligand recognition by the SRA, other rules may apply for recognition by alternate receptors for oxLDL. The modified LDL preparations reported here may serve as valuable ligands for molecular characterization of these novel receptor classes and their mechanisms of operation.

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